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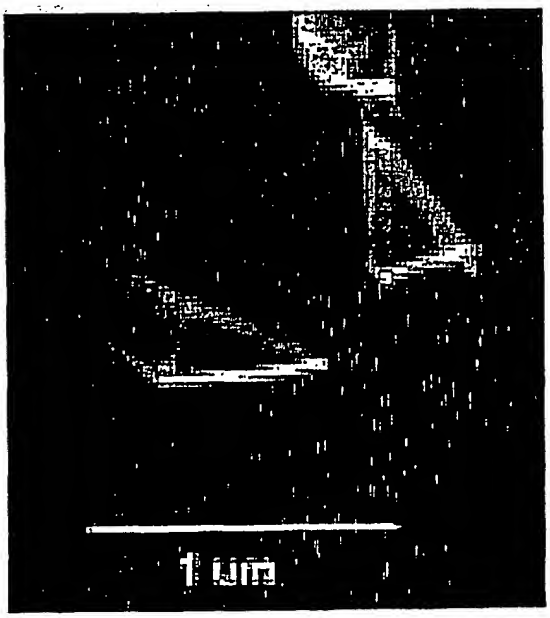
(54) Title: METHOD FOR SCREENING CRYSTALLIZATION CONDITIONS IN SOLUTION CRYSTAL GROWTH

(57) Abstract

A method of screening protein crystal growth conditions with picogram to microgram amounts of protein in picoliter or nanoliter volumes is provided. A preferred method comprises a microarray with a plurality of micro–chambers in the microarray. A protein solution is placed into the micro–chambers by an automated dispensing mechanism. The protein crystal growth conditions of each of the micro–chambers is adjusted so that the protein crystal growth conditions in at least two of the micro–chambers differs. Crystallization of the protein solution in the micro–chambers is effected. Protein crystal growth in the micro–chambers is then observed.



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thermal methods such as tyndallization or inspissation. These methods may be rendered more effective for some injections by the inclusion of a bacteriostatic agent in the product.

It should be obvious that all materials subjected to sterilization must be protected from subsequent contamination to maintain their sterile state. Therefore, materials subjected to autoclaving must be wrapped or covered so that microorganisms may not gain access when removed from the autoclave. Equipment and supplies are most frequently wrapped with paper and tied or sealed with special autoclave tape. The wrapping must permit penetration of steam during autoclaving but screen out microorganisms when dry. A double wrapping with lint-free parchment paper designed for such use is probably best. Synthetic fiber cloth such as nylon or Dacron also may be used for the inner wrapping. The openings of equipment subjected to dry-heat sterilization are often covered with silver–aluminum foil or with metal or glass covers. Cellulose wrapping materials are adversely affected by the high temperatures of dry-heat sterilization.

The effectiveness of any sterilization technique must be proved before it is employed; controls then must be established to show that subsequent processes repeat the conditions proven to be effective. Since the goal of sterilization is to kill microorganisms, the ideal indicator to prove the effectiveness of the process is a biological one, resistant spores. However, many feel considerable hesitation about using biological indicators during the processing of products because of the inherent risk of inadvertent contamination of the product or the environment. Also, it has been found that the resistance of spores varies from lot to lot, thereby possibly giving false indications of reliability when used as a biological indicator for a sterilization procedure. However, others feel as strongly that biological indicators should be used, not only to prove the effectiveness of a sterilization procedure but as confirmatory evidence for the effectiveness of each sterilization process.

It is also essential to utilize other indicators to confirm the reliability of the sterilization process, such as recording thermocouples, color-change indicators, and melting indicators. Such confirmatory evidence is an essential part of the sterilization record for a product.

Further details concerning methods of sterilization and their application will be found in Chapter 78. In addition, the USP provides suggestions concerning the sterilization of injections and related materials.

Freeze-Drying

Freeze-drying (lyophilization) is a process of drying in which water is sublimed from the product after it is frozen.[14]

The particular advantages of this process are that biologicals and pharmaceuticals which are relatively unstable in aqueous solution can be processed and filled into dosage containers in the liquid state, taking advantage of the relative ease of processing a liquid; dried without elevated temperatures, thereby eliminating adverse thermal effects; and then stored in the dry state in which there are relatively few stability problems.

Further advantages include the fact that freeze-dried products are often more soluble and/or more rapidly soluble, dispersions are stabilized throughout the shelf life of the product, and products subject to degradation by oxidation have enhanced stability because the process is carried out in a vacuum.

However, the increased time and handling required for processing and the cost of the equipment limit the use of this process to those products which have significantly enhanced stability if stored in the dry state.

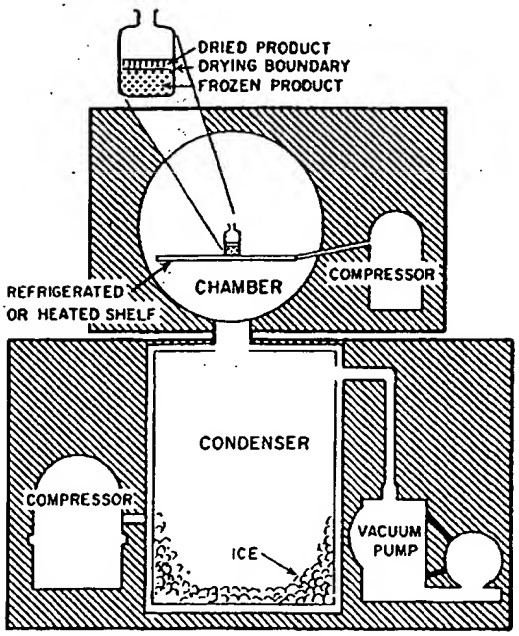

Fig. 84-30. Essential components of a freeze-drying system.

has been a long-established laboratory principle. The extensive program for the freeze-drying of human plasma during World War II provided the impetus for the rapid development of the process.

Freeze-drying essentially consists of the following:

1. Freezing an aqueous product at a temperature below its eutectic temperature.
2. Evacuating the chamber, usually below 0.1 torr (100 μm Hg).
3. Subliming ice on a cold condensing surface at a temperature below that of the product, the condensing surface being within the chamber or in a connecting chamber.
4. Introducing heat to the product under controlled conditions, thereby providing energy for sublimation at a rate designed to keep the product temperature below its eutectic temperature.

Fig. 84-30 shows such a system. The product may be frozen on the shelf in the chamber by circulating refrigerant (usually Freon, ammonia, or ethylene glycol) from the compressor through pipes within the shelf. After freezing is complete, which may require several hours, the chamber and condenser are evacuated by the vacuum pump, the condenser surface having been previously chilled by circulating refrigerant from the large compressor.

Heat is then introduced from the shelf to the product by electric resistance coils or by circulating hot water or hot glycol. The process continues until the product is dry (usually 1% or less moisture), leaving a sponge-like matrix of the solids originally present in the product, the input of heat being controlled so as not to degrade the product.

For most pharmaceuticals and biologicals the liquid product is sterilized by filtration and then filled into the dosage container aseptically. The containers must remain open during the drying process; therefore, they must be protected from contamination during transfer from the filling area to the freeze-drying chamber, while in the freeze-drying chamber, and at the end of the drying process until sealed.

Chambers may be equipped with hydraulic or rubber diaphragm internal-stoppering devices designed to push slotted rubber closures into the vials to be sealed while the chamber is still evacuated, the closures having been partially inserted immediately after filling so that the

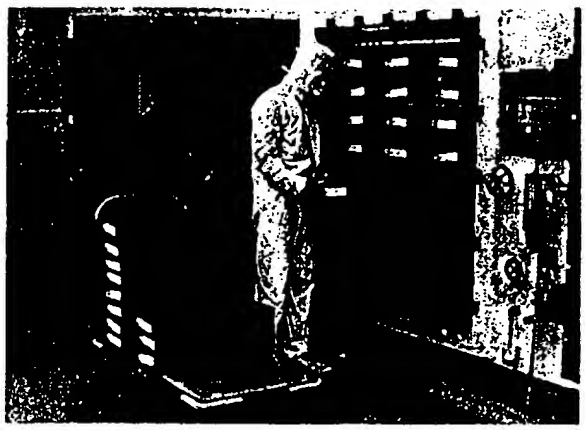

Fig. 84-31. Aseptic loading of freeze-drier (courtesy, Upjohn).

If internal stoppering is not available or containers such as ampuls are used, filtered dry air or nitrogen must be introduced to the chamber at the end of the process to establish atmospheric pressure. Then the containers must be removed and sealed under aseptic conditions. If the product is very sensitive to moisture, the environmental humidity also must be controlled until it is sealed.

Factors Affecting the Process Rate—The greater the depth of the product in the container, the longer will be the drying process. Therefore, a product to be frozen by placing the container on a refrigerated shelf (plug freezing) should be filled to a planned, limited depth. If a large volume of solution must be processed, the surface area may be increased and the depth decreased by freezing the solution on a slant or while rotating the container on an angle (shell freezing) in a liquid refrigerant bath, such as dry ice and alcohol.

The actual driving force for the process is the vapor pressure differential between the vapor at the surface where drying of the product is occurring (the drying boundary) and the vapor pressure at the surface of the ice on the condenser. The latter is determined by the temperature of the condenser as modified by the insulating effect of the accumulated ice. The former is determined by a number of factors, including:

1. The rate of heat conduction through the container and the frozen material, both usually relatively poor thermal conductors, to the drying boundary while maintaining all of the product below its eutectic temperature.
2. The impeding effect of the increasing depth of dried porous product above the drying boundary.
3. The temperature and heat capacity of the shelf itself.

This may be visualized by referring to Fig. 84-30.

The passageways between the product surface and the condenser surface must be wide open and direct for effective operation. Therefore, the condensing surfaces in large freeze-driers are usually in the same chamber as the product. Evacuation of the system is necessary to reduce the impeding effect that collisions with air molecules would have on the passage of water molecules. However, the residual pressure in the system must be greater than the vapor pressure of the ice on the condenser or the ice will be vaporized and pulled into the pump, an event detrimental to most pumps.

The amount of solids in the product, their particle size, and their thermal conductance will affect the rate of drying. The more solids present, the more impediment will be provided to the escape of the water vapor. The smaller the particle size, particularly the crystal size of the ice, the faster the drying generally will be. The poorer the thermal conducting properties of the solids in the product, the slower will be the rate of transfer of heat through the frozen material to the drying boundary.

The rate of drying is essentially slow, most often requiring 24 hours or longer for completion. The actual time required, the rate of heat input, and the product temperatures that may be utilized must be determined for each product and then carefully reproduced with successive processes.

Factors Affecting Formulation—The active constituent of many pharmaceutical products is present in such a small quantity that if freeze-dried alone its presence would be hard to detect visually. Therefore, substances are often added to increase the amount of solids present.

Some consider it ideal for the dried product plug to occupy essentially the same volume as that of the original solution. To achieve this, the solids content of the original product must be between approximately 10 and 25%. Among the substances found most useful for this purpose, usually as a combination, are sodium or potassium phosphates, citric acid, tartaric acid, gelatin, and carbohydrates such as dextrose, mannitol, and dextran.

Each of these substances contributes appearance characteristics to the plug, such as whether dull and spongy or sparkling and crystalline, firm or friable, expanded or shrunken, and uniform or striated. Therefore, the formulation of a product to be freeze-dried must include consideration not only of the nature and stability characteristics required during the liquid state, both freshly prepared and when reconstituted before use, but the characteristics desired in the dried plug.

Modifications in the Process and Equipment—In some instances a product may be frozen in a bulk container or in trays rather than in the final container and then handled as a dry solid. This may be desirable when large volumes of a product are processed.

Heat may be introduced to all sides of the product by radiation from infrared sources, rather than only from the bottom as with conductive heating. While this generally increases the rate of drying, there are at least two major disadvantages to radiant heating of pharmaceuticals; these are (1) multiple containers produce shadowing with resultant blockage of the radiations and (2) the dried material on the outside of the frozen product may be scorched easily by the heat as drying progresses.

When large quantities of material are processed it may be desirable to utilize ejection pumps in the equipment system. These draw the vapor into the pump and eject it to the outside, thereby eliminating the need for a condensing surface. Such pumps are expensive and usually practical only in large installations.

Available freeze-driers* range in size from small laboratory units to large industrial models such as those shown in Fig. 84-31. Their selection requires consideration of such factors as tray area required, volume of water to be removed, whether or not aseptic processing will be involved, is internal stoppering required, will separate freezers be used for initial freezing of the product, and the degree of automatic operation desired.

Freeze-drying is now being utilized for research in the preservation of human tissue and is finding increasing application in the food industry. Progress on new developments is being made in both the process and the equipment.

* Suppliers: *Hull, Industrial Dynamics, NRC, Repp, Stokes, Vacuum, Virtis.*

Quality Control

The importance of undertaking every possible means to be assured of the quality of the finished product cannot be overemphasized. Every component and every step of the manufacturing process must be subjected to intense scrutiny to be confident that quality is attained in the finished product. The responsibility for supervising this is a grave one, and lapses of requirements or short cuts in procedure may not be permitted. Such responsibility applies wherever parenteral preparations are manufactured.

The principles of quality control are basically the same for the manufacture of any pharmaceutical. These are discussed in Chapter 82. During the discussion of the preparation of injections, mention was made of numerous quality requirements for components and manufacturing processes. Here, only certain tests characteristically applicable to parenteral preparations will be discussed.

Sterility Test

All lots of injections in their final containers must be tested for sterility. The USP prescribes the requirements for this test for official injections. The Food and Drug Administration uses these requirements as a guide for testing unofficial sterile products. The official test has acknowledged limitations in the information that it can provide. Therefore, it should be noted that this test is not intended as a thoroughly evaluative test for a product subjected to a sterilization method of unknown effectiveness. It is intended primarily as a check test on the repetition of a previously proved sterilization procedure, or to give assurance of its continued effectiveness. A discussion of sterility testing is given in Chapter 78.

It should be noted that a "lot" with respect to sterility testing is that group of product containers which has been subjected to the same sterilization procedure. For containers of a product which have been sterilized by autoclaving, for example, a lot would constitute those processed in a particular sterilizer cycle. For an aseptic filling operation, a lot would constitute all of those product containers filled during a period when there was no change in the filling assembly or equipment and which is no longer than one working day or shift.

Pyrogen Test

The presence of pyrogens in parenteral preparations is evaluated by a qualitative fever response test in rabbits. The USP test is described in Chapter 31. Rabbits are used as test animals because they show physiologic response to pyrogenic substances similar to that by man. While a minimum pyrogenic dose (MPD), the amount just sufficient to cause a positive USP Pyrogen Test response, may sometimes produce uncertain test results, a content equal to a few times the MPD will leave no uncertainty. Therefore, the test is valid and has remained first in choice since introduced by Seibert in 1923. It should be understood that not all injections may be subjected to the pyrogen test since the medicinal agent may have a physiologic effect on the test animal such that any fever response would be masked. Therefore, the pyrogen test is performed primarily on vehicles.

A new test for pyrogens is receiving much favorable consideration. It is an *in vitro* test based on the gelling of a pyrogenic preparation in the presence of the lysate of the amebocytes of the horseshoe crab (*Limulus polyphemus*). The Limulus Test, as it is called, appears to be simpler, more rapid, and of greater sensitivity than the rabbit test.[15] Although it detects only the endotoxic pyrogens of gram-negative bacteria, this probably will not significantly limit its use since most environmental contaminants gaining entrance [illegible]

Clarity Tests

The USP does not provide specifications for a clarity test. It contains only the statement that good manufacturing practice requires that each final container of an injection should be subjected individually to a visual inspection. The development of test procedures to meet this general requirement is the responsibility of the manufacturer.

The objective of the clarity inspection is to prevent the distribution and use of parenterals which contain particulate matter that may be psychologically or actually harmful to the recipient. Solutions to be introduced intravenously require the most critical evaluation.

Until recently, concern about particulate matter in parenteral solutions was limited largely to the psychological effect on the user in that the presence of visible "dirt" would suggest that the product was of inferior quality; however, further investigation has caused a new assessment to be undertaken of the significance of particles in solutions to be introduced into the blood stream. While data defining the extent and risk of toxic effects is still rather nebulous, it has been shown that particles of lint, rubber, insoluble chemicals, and other foreign particulate matter can produce emboli in vital organs of animals and man. A recent study suggests that another adverse physiologic effect may be related to the presence of particulate matter in intravenous fluids, namely, the development of infusion phlebitis.

A study of the size distribution of particulate matter in commercial intravenous solutions showed that the number of particles increased approximately logarithmically with decreasing size. This finding would suggest that a count made at an arbitrarily chosen size could be used to predict the number of particles at another size. The counts were made with a Coulter Counter, a resistance-type counter. Other electronic counters utilize the light-scattering* or light-shadowing† principle to count particles in a liquid sample. Particles may also be counted and examined microscopically by collection on the surface of a membrane filter, a method that permits identification of the particles as well as a count. Methods of particulate evaluation such as these are performed on a sample from a container. Such methods cannot be utilized for the in-line evaluation of every container produced commercially, but may be used for quality-control sampling of the process.

The particle size that should be of particular concern has not been determined but it has been suggested that, since erythrocytes have a diameter of approximately 4.5 μm, particles of more than 5 μm should be the basis for evaluation. This is a considerably smaller particle than can be seen with the unaided eye; approximately 50 μm is the lower limit unless the Tyndall effect is utilized, whereby particles as small as 10 μm may be seen by the light scattered from them.

Meanwhile, the product units from the production line are being inspected individually by human inspectors under a good light, baffled against reflection into the eye, against a black and a white background. Although this inspection is subject to the limitations in the size of particles that can be seen, the variation in visual acuity from inspector to inspector, the emotional state of the inspector, eye strain and fatigue, and other personal factors that will affect what the inspector sees, it does provide a means for eliminating the normally few units which contain visible particles and it is a check on the repetition of the standard clean processing procedure established for that product.

This concern over the presence of particulate matter in parenteral products, particularly those given intravenously, has brought about a dramatic improvement through the voluntary effort of the pharmaceutical industry. One study[16] clearly shows a reduced particulate content of commercially prepared intravenous infusion fluids, as compared with earlier work. Also, it has been shown that additives and administration sets may introduce a substantial amount of particulate matter to an otherwise relatively clean solution. In addition, the technique utilized in the hospital for the preparation and administration of the intravenous infusion fluid must be carefully controlled to avoid the introduction of particulate matter. Therefore, the pharmaceutical manufacturer, the administration set manufacturer, the hospital pharmacist, the nurse, and the physician must share responsibility for making sure that the patient receives a clean intravenous injection.

Leaker Test

Ampuls that have been sealed by fusion must be subjected to a test to determine whether or not a passageway remains to the outside. If such a passageway remains, all or a part of the contents of the ampul may leak to the outside and spoil the package, or microorganisms or other contaminants may enter. Changes in temperature during storage cause expansion and contraction of the ampul and contents, and will accentuate interchange if a passageway exists.

A leaker test is usually performed by producing a negative pressure within an incompletely sealed ampul while the ampul is entirely submerged in a deeply colored dye solution. Most often, approximately a 1% methylene blue solution is employed. The test may be performed by subjecting the ampuls to a vacuum in a vacuum chamber, the ampuls being submerged in a dye bath throughout the process. Another procedure frequently employed is to simply autoclave the ampuls in a dye bath. A modification of this is to remove them from the autoclave while hot and quickly submerge them in a cool bath of dye solution. After carefully rinsing the dye solution from the outside, color from the dye will be visible within a leaker. Leakers are, of course, discarded.

Vials and bottles are not subjected to a leaker test because the sealing material (rubber stoppers) is not rigid. Therefore, results from such a test would be meaningless. However, evacuated bottles containing a liquid may be checked for a sharp "click" sound produced when struck with an implement such as a rubber mallet.

Safety Test

The National Institutes of Health requires of most biological products routine safety testing in animals. Under the Kefauver-Harris Amendments to the Federal Food, Drug, and Cosmetic Act, most pharmaceutical preparations are now required to be tested for safety. Because it is entirely possible for a parenteral product to pass the routine sterility test, pyrogen test, and chemical analyses and still cause unfavorable reactions when injected, a safety test in animals is essential to provide additional assurance that the product does not have unexpected toxic properties. Safety tests in animals are discussed in detail in the USP.

Packaging and Labeling

A full discussion of the packaging of parenteral preparations is beyond the scope of this text. It is essential, of course, that the packaging should provide ample protection for the product against physical damage from shipping, handling, and storage and should protect light-sensitive materials from ultraviolet radiation.

Packaging—The USP includes certain requirements for the packaging and storage of injections, as follows:

1. The volume of injection in single-dose containers is defined as that which is specified for parenteral administration at one time and is limited to a volume of 1 liter.
2. Parenterals intended for intraspinal, intracisternal, or peridural administration are packaged only in single-dose containers.
3. Unless an individual monograph specifies otherwise, no multiple-dose container shall contain a volume of injection more than sufficient to permit the withdrawal and administration of 30 ml.
4. Injections packaged for use as irrigation solutions or for hemofiltration or dialysis are exempt from foregoing requirements relating to packaging. Containers for injections packaged for use as hemofiltration or irrigation solutions may be designed to empty rapidly and may contain a volume in excess of 1 liter.
5. Injections intended for veterinary use are exempt from the packaging and storage requirements concerning the limitation to single-dose containers and to volume of multiple-dose containers.

Labeling—The labeling of an injection must provide the physician or other user with all of the information needed to assure the safe and proper use of the therapeutic agent. Since all of this information cannot be placed on the immediate container and be legible, it may be provided on accompanying printed matter. General labeling requirements for drugs are discussed in Chapter 106.

A restatement of the labeling definitions and requirements of the USP for Injections is as follows:

The term "labeling" designates all labels and other written, printed, or graphic matter upon an immediate container or upon, or in, any package or wrapper in which it is enclosed, with the exception of the outer shipping container. The term "label" designates that part of the labeling upon the immediate container.

The label states the name of the preparation, the percentage content of drug of a liquid preparation, the amount of active ingredient of a dry preparation, the volume of liquid to be added to prepare an injection or suspension from a dry preparation, the route of administration, a statement of storage conditions, and an expiration date. Also, the label must indicate the name of the manufacturer or distributor and carry an identifying lot number. The lot number is capable of providing access to the complete manufacturing history of the specific package, including each single manufacturing step.

The container label is so arranged that a sufficient area of the container remains uncovered for its full length or circumference to permit inspection of the contents.

The label must state the name of the vehicle and the proportions of each constituent, if it is a mixture; the names and proportions of all substances added to increase stability or usefulness; and the expiration date where required by the individual monograph.

Preparations labeled for use as irrigating solutions must meet the requirements for Injections other than those relating to volume and also must bear on the label statements that they are not intended for intravenous injection.

Injections intended for veterinary use are so labeled.

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Chapter 85

Intravenous Admixtures

Intravenous fluids
packaging
systems
administration
sets
administration
procedures
admixtures
total parenteral
nutrition
parenteral
prescriptions

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It has been estimated that 40% of all drugs administered in hospitals are given in the form of injections and their use is increasing. Part of this increase in parenteral therapy is due to the wider utilization of intravenous fluids (IV fluids). In the last decade the use of IV fluids has doubled, increasing from 150 million units to 250 million units annually. Not only do IV fluids continue to serve as the means for fluid replacement, electrolyte-balance restoration, and supplementary nutrition, but they are also playing major roles as vehicles for other drug substances and in total parenteral nutrition. Intravenous fluids are finding greater use as the means of administering other drugs because of convenience, the means of reducing the irritation potential of the drugs, and the desirability for continuous and intermittent drug therapy. The techniques for providing total parenteral nutrition parenterally have improved steadily in the last decade and such use is increasing at the rate of 40% annually. The use of IV fluids for these purposes requires the compounding of specific intravenous admixtures (parenteral prescriptions) to meet the clinical needs of a given patient. However, the combination of drug substances in an intravenous fluid can promote parenteral incompatibilities and give rise to conditions not favorable for drug stability. A new area of specialization has been created for hospital pharmacists who can develop the expertise to prepare these solutions, recognizing their compatibility and stability problems and the potential for contamination, and to participate in the administration of the solutions. The complex compounding of an order for total parenteral nutrition requires knowledgeable personnel capable of making accurate calculations, compounding, and having perfect aseptic technique. The parenteral prescription is becoming increasingly important in hospitals. Centralized admixture programs are now found in 50% of the nation's hospitals having 300 beds or more. Equipment available for administering intravenous fluids has become more sophisticated, and has made possible increased accuracy of dosage and led to the development of new concepts and methods of nutrition and drug treatment.

Intravenous Fluids

Large-volume injections intended to be administered by intravenous infusion are commonly called IV fluids and are included in the group of sterile products referred to as large-volume parenterals. Large-volume parenterals consist of single-dose injections having a volume of 100 ml or more and containing no added substances. Intravenous fluids are packaged in containers having a capacity of 150 ml to 1000 ml. Minitype infusion containers of 250-ml capacity are available with 50- and 100-ml partial fills for solution of drugs when used in the "piggyback" technique. This technique refers to the administration of a second solution through a Y-tube or gum-rubber connection in the administration set of the first intravenous fluid, thus avoiding the need for another injection site. In addition to the IV fluids, the group also includes irrigation solutions and solutions for dialysis.

Intravenous fluids are sterile solutions of simple chemicals such as sugars, amino acids, or electrolytes—materials which can easily be carried by the circulatory system and assimilated. Prepared with Water for Injection USP, the solutions are pyrogen-free. Because of the large volumes administered intravenously, the absence of particulate matter assumes a more significant role in view of possible biological hazards resulting from particulate matter. Absence of particulate matter or clarity of IV fluids is as important at the time of administration following their manipulation in the hospital as it is at the time of injection manufacture.

Limits for particulate matter occurring in IV fluids, or large-volume injections used for single-dose infusion, have been defined in the USP. This represents the first regulatory attempt to define limits for particulate matter in parenterals. These limits do not apply to multiple-dose injections, small-volume injections, or injections prepared by reconstitution from sterile solids. The USP defines particulate matter as extraneous, mobile, undissolved substances, other than gas bubbles, unintentionally present in parenteral solutions. The microscopic membrane method is used for determining the presence and size distribution of the particles observed. The determination is carried out in a laminar airflow hood using ultraclean equipment. The IV fluid sample is placed in an ultraclean funnel containing an ultraclean grid membrane through which the sample passes. After the particulate matter is collected on the membrane, the membrane is rinsed and dried within the hood. The entire surface of the membrane is examined within the hood, using a suitable microscope under 100× magnification. The total numbers of particles having effective linear dimensions equal to or larger than 10.0 μm and larger than 25.0 μm are counted. The fluid meets the requirement of the test if it contains not more than 50 particles per ml that are equal to or larger than 10 μm, and not more than 5 particles per ml that are equal to or larger than 25.0 μm in linear dimensions.

Intravenous fluids are commonly used for a number of clinical conditions. These include: (1) correction of disturbances in electrolyte balance; (2) correction of disturbances in body fluids (fluid replacement); (3) the means of providing basic nutrition; (4) the basis for the practice of providing total parenteral nutrition (TPN) or parenteral hyperalimentation; and (5) use as vehicles for other drug substances. In both the latter two cases it has become common practice to add other drugs to certain IV fluids to meet the clinical needs

Table I—IV Fluids Commonly Used For Intravenous Admixtures

Injection	Concentration	pH	Therapeutic Use
Amino Acid (Synthetic)			Fluid and nutrient replenisher
Aminosyn (Abbott)	3.5%; 7%	5.25	
FreAmine II (McGaw)	8.5%	6.6	
Travasol (Travenol)	5.5%; 8.5%	6.0	
Veinamine (Cutter)	8%	6.2–6.6	
Dextrose (Glucose, D5/W)	2.5%–50%	3.5–6.5	Fluid and nutrient replenisher
Dextrose and Sodium Chloride	Varying concn of dextrose from 5%–20% with varying concn of sodium chloride from 0.11%–0.9%	3.5–6.5	Fluid, nutrient, and electrolyte replenisher
Fructose (Levulose)	10%	3.0–6.0	Fluid and nutrient replenisher
Fructose and Sodium Chloride	10% 0.9%	3.0–6.0	Fluid, nutrient, and electrolyte replenisher
Invert Sugar	5%, 10%	4.0	Fluid and nutrient replenisher
Lactated Ringer's (Hartmann's)		6.0–7.5	Systemic alkalizer; fluid and electrolyte replenisher
NaCl	0.6%		
KCl	0.03%		
$CaCl_2$	0.02%		
Na Lactate	0.3%		
Protein Hydrolysate Amigen (Travenol) Aminosol (Abbott) CPH-5 (Cutter) Hyprotigen (McGaw)	5% from either casein or fibrin	5.0–7.0	Fluid and nutrient replenisher
Ringer's		5.0–7.5	Fluid and electrolyte replenisher
NaCl	0.86%		
KCl	0.03%		
$CaCl_2$	0.033%		
Sodium Chloride	0.45%; 0.9%; 3%; 5%	4.5–7.0	Fluid and electrolyte replenisher
Sodium Lactate	1/6 M	6.3–7.3	Fluid and electrolyte replenisher

the patient. Using IV fluids as vehicles offers the advantages of convenience, the means of reducing the irritation potential of the drug, and provides a method for continuous drug therapy. However, the practice requires that careful consideration be given to the stability and compatibility of additives present in the IV fluids serving as vehicles. This approach also demands strict adherence to aseptic techniques in adding the drugs, as well as in the administration of the IV fluids. These procedures are discussed later in the chapter. The IV fluids commonly used for parenteral admixtures are shown in Table I.

Many disease states result in electrolyte depletion and loss. Proper electrolyte concentration and balance in plasma and tissues are critical for proper body function. Electrolyte restoration and balance are most rapidly achieved through administration of IV fluids. Required electrolytes include sodium and chloride ions, which in normal saline more closely approximate the composition of the extracellular fluid than solutions of any other single salt; potassium, the principal intracellular cation of most body tissues and an essential for the functioning of the nervous and muscular systems as well as the heart; magnesium, as a nutritional supplement especially in hyperalimentation solutions; and phosphate ion, important in a variety of biochemical reactions. In addition to the number of standard electrolyte fluids shown in Table I, a large number of combinations of electrolytes in varying

either for nutrition or fluid replacement. It is isotonic and administered intravenously into a peripheral vein. One gram of dextrose provides 3.4 Calories and a liter of Dextrose Injection 5% supplies 170 Calories. The body utilizes dextrose at a rate of 0.5 g per kilogram of body weight per hour. More rapid administration can result in glycosuria. Therefore a liter of Dextrose Injection 5% requires one and one-half hours for assimilation. The pH range of Dextrose Injection 5% can vary from 3.5 to 6.5. The wide range permitted is due to the free sugar acids present and formed during the sterilization and storage of the injection. To avoid incompatibilities when other drug substances are added to Dextrose Injection, the possible low pH should be considered in using it as a vehicle. More concentrated solutions of dextrose are available and provide increased calorie intake with less fluid volume. Being hypertonic, the more concentrated solutions may be irritating to peripheral veins. Highly concentrated solutions are administered only in a larger central vein. Other IV fluids used for intravenous admixtures and providing calories include solutions of fructose (levulose) and those containing invert sugar. There is some evidence that fructose, unlike dextrose, may be used in diabetic patients; the 10% injection is hypertonic and provides 375 calories per liter. Invert sugar consists of equal parts of dextrose and levulose; it is claimed that the presence of levulose promotes more rapid utilization of dextrose.

synthesis (Chapter 52, p. 970). Protein contributes to tissue growth, wound repair, and resistance to infection. The protein requirement for the normal adult is 1 g per kilogram of body weight per day; children and patients under stress require greater amounts. Attempts are made to maintain a positive nitrogen balance, indicating that the protein administered is being properly utilized and not broken down and eliminated through the urine as creatinine and urea, which are normal waste products. In positive nitrogen balance the patient is taking in more nitrogen than he is eliminating. In negative nitrogen balance there is more nitrogen being eliminated through the urine regularly than is being administered intravenously. This means that tissues are continuing to be torn down and repair is not necessarily taking place. Protein Hydrolysate Injection and Amino Acid Injection can afford the total body requirements for proteins by the procedure known as total parenteral nutrition (discussed below), or be used for supplemental nutrition by peripheral administration. In addition to the amino acids or peptides, these nutritional injections may also contain dextrose, electrolytes, vitamins, and insulin. Fat emulsion (*Intralipid*, Cutter; *Liposyn*, Abbott) is sometimes used concurrently but administered at another site.

Packaging Systems

Containers for intravenous fluids must be designed to maintain solution sterility, clarity (freedom from particulate matter), and nonpyrogenicity from the time of preparation, through storage, and during clinical administration. Container closures must be designed to facilitate insertion of administration sets through which the injections are administered at a regulated flow-rate into suitable veins. IV fluids are available in glass and plastic containers; the latter may be made from either a flexible or semirigid plastic material. IV fluids are supplied in 1000-ml, 500-ml, and 250-ml sizes in addition to 250-ml capacity containers packaged with 50 or 100 ml of Dextrose Injection 5% or Sodium Chloride Injection for piggyback use. IV fluids in glass containers are packaged under vacuum, which must be dissipated prior to use. For fluid to leave the IV glass container and flow through the administration set, some mechanism is necessary to permit air to enter the container. Current flexible plastic systems do not require air introduction in order to function. Atmospheric pressure pressing on the container forces the fluid to flow.

All glass and plastic containers are single-dose and should be discarded if not used after opening. Intravenous fluids are packaged with approximately 3% excess fill to allow for removal of air from the administration set and permit the labeled volume to be delivered from the container. The containers are graduated at 20-ml increments on scales that permit the volume in container to be determined either from an upright or inverted position. Glass containers have aluminum bands for hanging while plastic containers have eyelet openings or plastic straps for attachment to IV poles.

IV fluids are available from four sources. With the exception of those from Cutter Laboratories, all provide both glass and plastic containers. The glass-container systems of Travenol/Baxter and McGaw are similar, as are the system designs of Abbott and Cutter Laboratories. The characteristics of current packaging systems are summarized in Table II.

Table II—IV Fluid Systems

Source	Container	Characteristics
Travenol/Baxter	Glass	Vacuum Air tube
Travenol/Baxter (Viaflex®)	Plastic	Polyvinyl chloride Flexible Non-vented
McGaw	Glass	Vacuum Air tube
McGaw (Accumed®)	Plastic	Polyolefin Semirigid Non-vented
Abbott	Glass	Vacuum Air filter*
Abbott (Lifecare®)	Plastic	Polyvinyl chloride Flexible Non-vented
Cutter	Glass	Vacuum Air filter*

* Part of administration set.

Administration Sets

Administration sets used to deliver fluids intravenously are sterile, pyrogen-free, and disposable. Although these sets are supplied by different manufacturers, each for its own system, they have certain basic components. These include a plastic spike to pierce the rubber closure or plastic seal on the IV container; a drip (sight) chamber to trap air and to permit adjustment of flow rate; and a length (60 to 180 in) of polyvinyl chloride tubing terminating in a gum-rubber injection port. At the tip of the port is a rigid needle or catheter adapter. An adjustable clamp (screw or roller type) on the tubing pinches the tubing to regulate flow. Since the gum-rubber port is self-sealing, additional medication can be added to the IV system at these ports of entry. Glass containers that have no

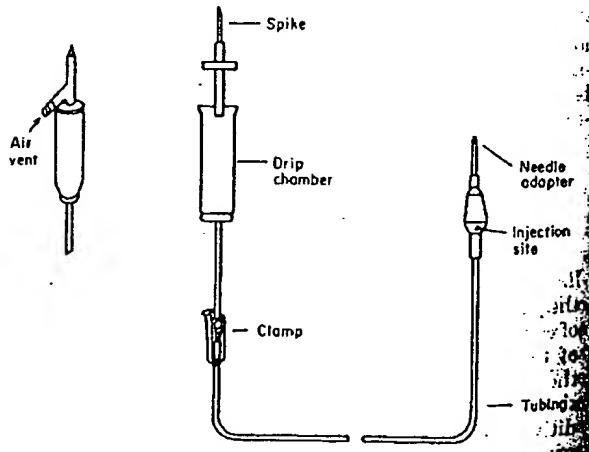

Fig. 85-1. Parts of basic administration sets.

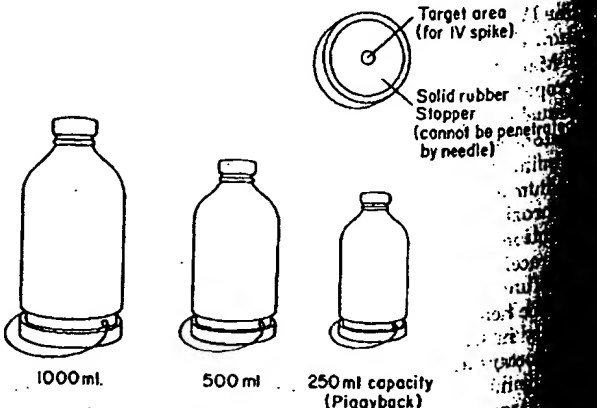

Fig. 85-2. Abbott and Cutter IV glass container. The air ve... provided through the air filter located in the spike of the adminis... set. See Fig. 85-1.

air tubes require air-inlet filters designed as part of the administration set (Abbott, Cutter). See Figs. 85-1 to 85-5.

Administration Procedures

In the administration of IV fluids, the primary IV container provides for fluid replacement, electrolyte replenishment, drug therapy, or nutrition; the fluid can be infused over a 4- to 8-hour period. In some cases an IV fluid is slowly infused for the purpose of keeping the vein open (KVO). This will allow additional drugs to be administered when required. The primary IV fluid can also serve as a vehicle for other drugs to be administered. This would then become an intravenous admixture (IV drip) and results in continuous blood levels of added drugs once the steady-state has been reached.

In preparing an IV fluid for administration, the following procedure is used.

1. The spike adapter of the administration set is inserted into stopper or seal of the IV container. Fig. 85-5.
2. The IV fluid is hung on a stand at bedside and air is purged from the administration set by opening clamp until fluid comes out of needle. The tubing is then clamped off. Fig. 85-5.
3. The venipuncture is made by member of the IV team, floor nurse or physician.
4. The infusion rate is adjusted by slowly opening and closing clamp until the desired drop rate, viewed in the drip chamber, is obtained. The usual running time is 4 to 8 hours (usually 125 ml are delivered in one hour). Drugs such as heparin, insulin, lidocaine, and dopamine may be present in the IV drip. When potent drugs are present, the flow-rates will vary and be dependent on the clinical condition of the patient. Sets are calculated to deliver 10, 15, 20, 50 or 60 drops per ml depending on manufacturer. Fig. 85-5.

Intermittent administration of an antibiotic and other drugs can be achieved by any of three methods. These are by (1) direct intravenous injection (IV bolus or push), (2) addition of the drug to a predetermined volume of fluid in a volume-control device, or (3) use of a second container (minibottle, minibag) with an already hanging IV fluid (piggybacking).

Direct Intravenous Injection—Small volumes (1 to 50 ml) of drugs are injected into the vein over a short period of time (1 to 5 minutes). The injection can also be made through a resealable gum-rubber injection site of an already hanging IV fluid. This method is suitable for a limited number of drugs but too hazardous for most drugs.

Volume-Control Method—Volume-control sets provide a means for intermittent infusion of drug solutions in precise quantities, at controlled rates of flow. These units consist of calibrated plastic fluid-chambers placed in a direct line under an established primary IV container or more often attached to an independent fluid supply. In either case, the drug to be administered is first reconstituted if it is a sterile solid and injected into the gum-rubber injection port of the volume-control unit. It is then further diluted to 50 to 150 ml with the primary fluid or the separate fluid reservoir. Administration of the total drug-containing solution requires 30 to 60 minutes and produces a peak concentration in the blood followed by a valley if the dosage is discontinued. The following volume control sets are available commercially: *Soluset®*, Abbott; *Buretrol®*, Travenol/Baxter; *Metriset®*, McGaw; and *Volutrol®*, Cutter.

The procedure for setting up an intermittent IV infusion with a volume-control set is as follows:

1. Using aseptic technique, the spike of the volume control set is inserted into the primary IV fluid or a separate fluid container. See Fig. 85-7.
2. Air is purged from tubing of the volume control set by opening clamps until fluid comes through. See Fig.
3. The clamp is opened above the calibrated chamber and it is filled with 25 to 50 ml fluid from the primary IV container or separate fluid container.

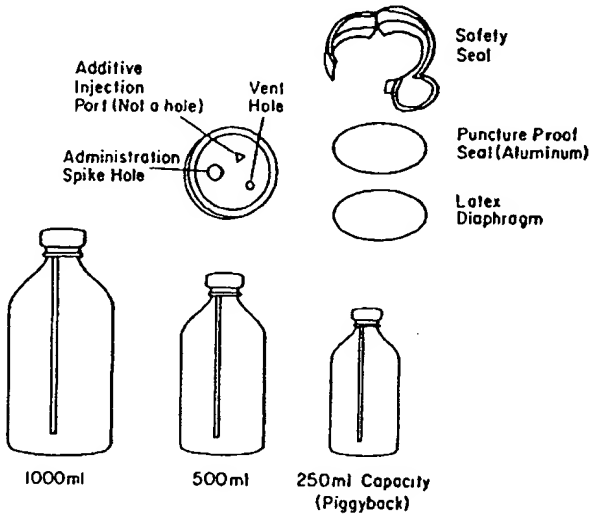

Fig. 85-3. Travenol/Baxter and McGaw glass container. The plastic air tube allows the air to enter the bottle as the fluid is infused into the patient. The spike of the administration set is not vented. See Fig. 85-1.

6. The clamp above the chamber is opened to complete the dilution to the desired volume (50 to 150 ml), then closed.
7. Flow commences when clamp below volume-control unit is opened.

Piggyback Method—The piggyback method refers to the intermittent intravenous drip of a second solution, the reconstituted drug, through the venipuncture site of an estab-

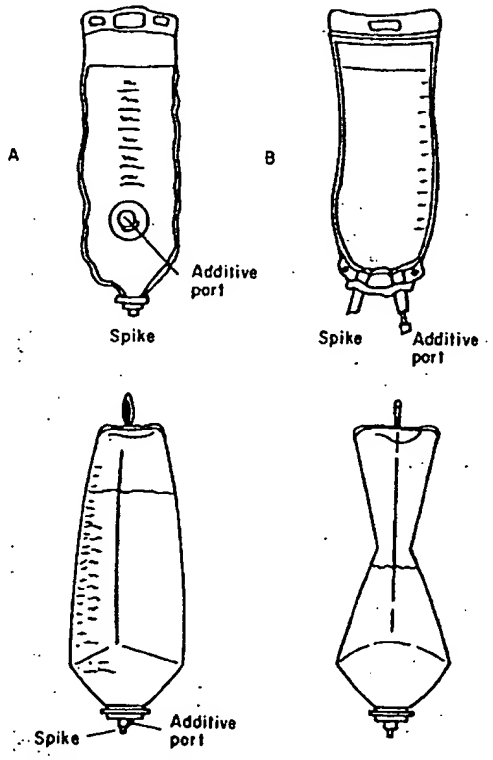

Fig. 85-4. (A) Abbott (Lifecare®) polyvinyl chloride flexible container; (B) Travenol/Baxter (Viaflex®) polyvinyl chloride flexible container;

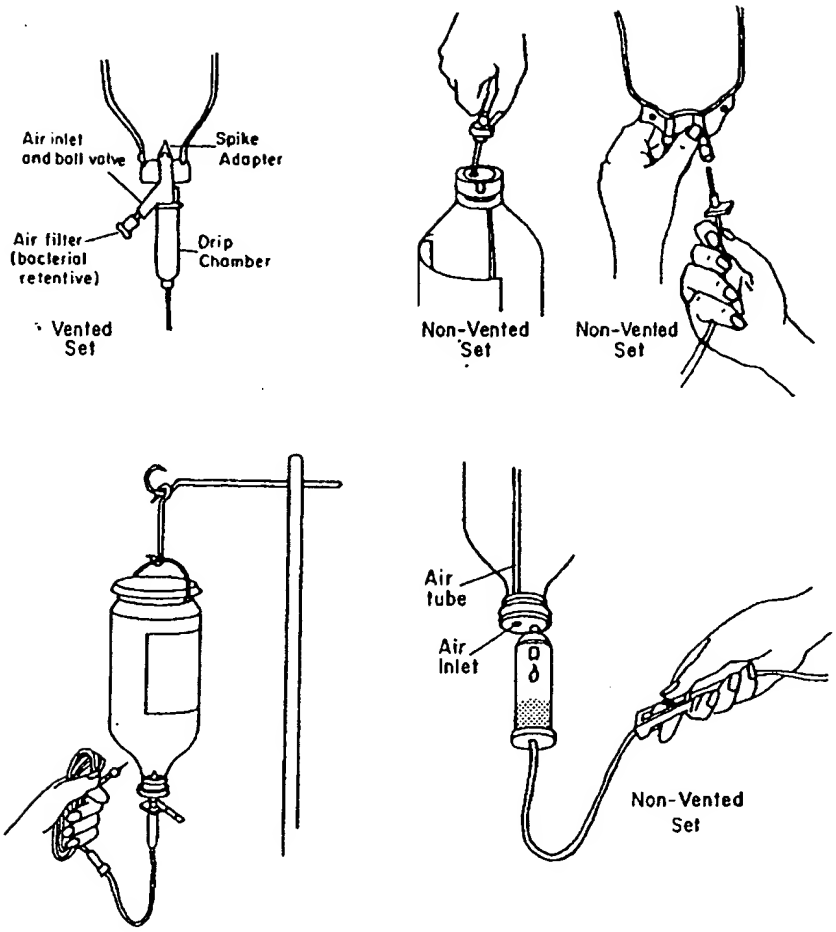

Fig. 85-5. Setting up primary IV fluid for administration.

Fig. 85-6. Piggyback method: the intermittent administration of a second solution through the venipuncture site of an established primary IV system.

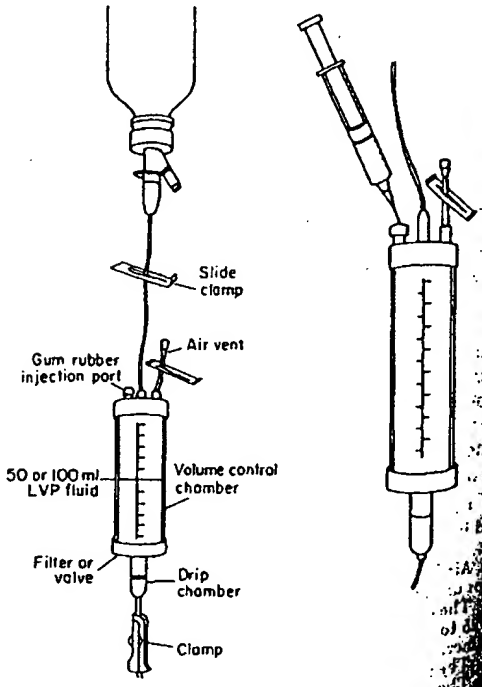

Fig. 85-7. Volume control set.

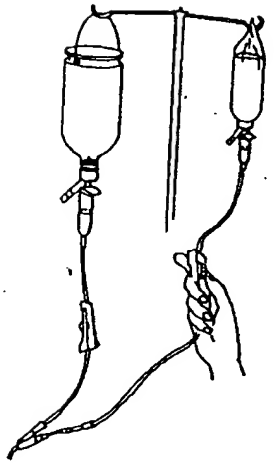

Fig. 85-8. Piggyback administration setup.

lished primary IV system. With this setup the drug can be thought of as entering the vein on "top" of the primary IV fluid, hence the designation "piggyback." The piggyback technique not only eliminates the need for another venipuncture, but also achieves drug dilution and peak blood levels within a relatively short time span, usually 30 to 60 minutes. Drug dilution helps to reduce irritation, and early high serum levels are an important consideration in serious infection requiring aggressive drug therapy. These advantages have popularized the piggyback method of IV therapy, especially for the intermittent administration of antibiotics. In using the piggyback technique, the secondary unit is purged of air and its needle inserted into a Y-injection site of the primary set or into the injection site at the end of the primary set. The piggyback infusion is then started. Once it is completed, the primary fluid infusion will be restarted. See Fig. 85-8.

Primary IV administration sets are available that have a built-in checkvalve for use in piggyback administration. When the piggyback is connected to one of these sets and started, the checkvalve automatically closes off the primary infusion. When the piggyback runs out, the check valve automatically opens, thereby restarting the primary infusion. The checkvalve works because of pressure differences. To achieve this difference, the primary container is hung lower than the secondary bottle by means of an extension hanger. See Fig. 85-9.

Several manufacturers have introduced minibottles prefilled with various antibiotic products; each container is provided with either a plastic bag or plastic hanger for direct suspension from an IV pole as the piggyback solution is administered through the resealable gum-rubber injection site of Y-type facility of an existing IV system. Reconstitution of piggyback units requires only the addition of a small volume of compatible diluent. Since reconstitution and administration proceed from the same bottle, no drug transfer is involved, so transfer syringes and additional IV containers are not necessary. Therefore prefilled piggyback units offer a greater ease in handling and a considerable reduction in inventory costs.

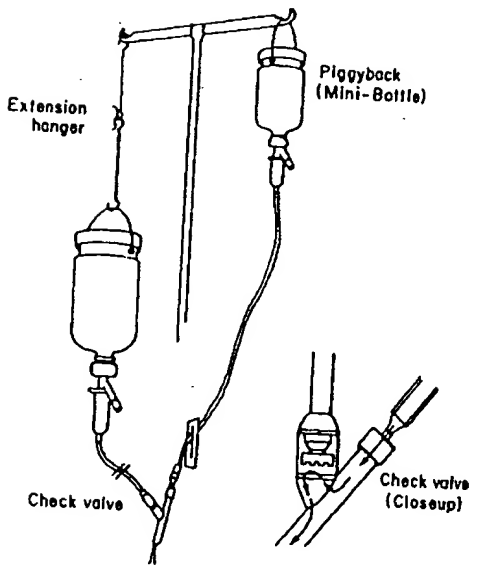

Fig. 85-9. Piggyback administration setup with check valve in primary set.

Partial-fill containers available for piggybacking are 250-ml capacity infusion bottles or bags underfilled with 50 or 100 ml Dextrose Injection, 5% or normal saline. The drug to be administered is first reconstituted in its original parenteral vial and then added by needle and syringe to the partial-fill container. The needle of the piggyback delivery system is inserted into the Y-site or gum-rubber injection port of a hanging primary infusion set. Flow of the primary intravenous fluid is stopped while the drug solution in the partial-fill container is administered (30 to 60 minutes). After the drug solution has been totally infused, the primary fluid flow is reestablished. When the next dose of drug is required, the piggyback procedure is repeated, replacing the prefilled partial-fill container.

Final Filter Devices—Particulate matter in intravenous fluids and intravenous admixtures can originate from many sources. It can result from the packaging components of the IV fluid, from admixture incompatibilities, from manipulation in preparing the admixture, and even from the administration set itself. Concern for particulate matter led to the design of final filter devices for attaching to the end of the tubing of the administration set. They afford a final filtration of the IV fluid before it passes through the needle into the vein. The device consists of a plastic chamber containing a membrane or stainless steel filter having porosities varying from 5 to 0.22 μm. Air lock can be a problem with membrane filters. When wet, membranes with a porosity of 0.22 μm and 0.45 μm are impervious to air at normal pressures and air in the system causes blockage. In order to prevent this, the filter housing must be completely purged of air prior to use. Newer device designs have air eliminators. Using final filter devices increases medication cost but reduces the biological hazards associated with particulate matter.

Intravenous Admixtures

When one or more sterile products are added to an IV fluid [illegible] administration, the resulting [illegible] listed in [illegible]

Fig. 85-10. Laminar flow hoods provide the proper environment for compounding IV admixtures.

in a parallel flow configuration at a velocity of 90 ft per minute. HEPA filters remove 99.97% of all particles larger than 0.3 μm. Since microbial contaminants present in air are usually found on other particulates, removal of the latter results in a flow of air free of both microbial contaminants and particulate matter. The movement of the filtered air in a laminar flow configuration at a velocity of 90 ft per minute can maintain the area free of contamination. The flow of air may be either in a horizontal or vertical pattern. In the former case the HEPA filter is located at the back of the hood and the air flows to the front. In vertical flow the air passes through the HEPA filter located in the top of the cabinet and is exhausted through a grated area around the working surface of the hood. Regardless of the type of laminar airflow, the hood must be properly operated and maintained in order to achieve a satisfactory environment for preparing parenteral admixtures.

The hood is best situated in a clean area in which there is little traffic flow past the front of the hood. The inside of the hood is thoroughly wiped down with a suitable disinfectant and allowed to run for at least 30 minutes before starting manipulations. It is important to remember that the laminar flow hood is not a means of sterilization. It only maintains an area free of microbial contaminants and particulate matter when it has been properly prepared, properly maintained, and utilized by operators having proper aseptic techniques.

Before working in a laminar airflow hood the operator washes his hands thoroughly and scrubs them with a suitable disinfectant. Some laboratories may require gowning and use of sterile gloves. Sterile gloves can be an asset but there is always the problem that they can give the operator a false sense of security. Gloved hands can become contaminated as easily as ungloved hands. Additives and IV fluids to be used in the preparation of the admixture, along with suitable syringes, are lined up in the hood in the order they are going to be used. The containers must be clean and dust free. They are inspected for clarity and freedom from cracks. Operators are encouraged to use a lighting device for inspecting IV fluids for particulate matter and cracks. The lighting device should be of the type that permits the container to be viewed against both a light and a dark background during inspection. If the IV fluid is packaged in plastic containers, pressure is applied to assure that they are properly sealed and do not leak. Some laboratories disinfect the containers prior to placing them in the hood.

In working within the hood the operator works in the center of the hood with the space between the point of operation and the filter unobstructed. If the flow of air is blocked, then the validity of the laminar flow is destroyed. Articles are arranged within the hood in a manner to prevent clean air from washing over dirty objects and contaminating other objects that must remain sterile. The working area must be at least six inches from the front edge of the hood. As the operator stands in front of the hood, his body acts as a barrier to the laminar air flow causing it to pass around him and create backflow patterns which can carry room air into the front of the hood.

Laminar flow hoods must be maintained and evaluated periodically to insure that they are functioning properly. The velocity of air flow can be determined routinely using a velometer. Decrease in the air flow usually indicates a clogged HEPA filter. Some laminar flow hoods are equipped with pressure gauges indicating pressure in the plenum behind the filter; in these hoods pressure increase can also indicate a clogged filter. Settling plates can be exposed within the hood for given periods of time to determine the presence of microbial contaminants.

The best way to determine the proper functioning of a HEPA filter is to use the dioctyl phthalate (DOP) test. DOP is a vapor at room temperature, its vapor particles being in the range of 0.3 μm. DOP vapor is allowed to be taken up by the hood through its intake filter. If the HEPA filter is intact and properly installed, no DOP can be detected in the filtered air stream using a smoke photometer. Certification services are available through commercial laboratories; the HEPA filters within laminar flow hoods should be evaluated every six months.

Additives—The additives are injections packaged in ampuls or vials, or sterile solids; the latter are reconstituted with a suitable diluent before addition to the IV fluid. A fresh, sterile, disposable syringe is used for each additive. Before removing a measured volume from an ampul, the container is wiped with a disinfectant solution. If the ampul is scored, the top can be snapped off; if not scored, an ampul file must be used. A sterile syringe is removed from its protective wrapping. The syringe needle with its cover is separated from the syringe aseptically and may be replaced with a sterile aspirating needle. Aspirating needles are usually made from clear plastic and contain a stainless steel or nylon filter having a porosity of 5 μm. The filter will remove glass particles and other particulates from the injection as it is drawn up from the ampul into the syringe. The aspirating needle is replaced with the regular needle. The exact volume is calibrated and the injection is ready to be added to the IV fluid (see Fig. 85-11). In the case of additives packaged in multiple-dose vials, the protective cover is removed and the exposed target area of the rubber closure disinfected. A volume of air, equal to the volume of solution to be removed, is drawn up into the syringe and injected into the air space above the injection within the vial. This will facilitate withdrawal of the injection. The solution is drawn into the syringe, the exact dose is measured, and the injection is ready to be added to the IV fluid.

In the case of drug substances having poor stability in aqueous solution, the drug is packaged as a sterile solid, either dry-filled or lyophilized. The diluent recommended on the labeling is used to reconstitute the powder; the proper quantity of solution is then removed for addition to the IV fluid. When large volumes of diluent are required for reconstitution as for Keflin 4 g, a sterile needle is placed through the closure to vent the container and facilitate addition of the diluent.

The procedure for placing an additive in an IV fluid will vary depending on the type of IV fluid packaging system being used by the hospital. The packaging systems have been described in Table II.

Abbott and Cutter Rigid Glass Containers (Fig. 85-2)

1. Remove the aluminum tear seal exposing the solid rubber closure with a target circle in the center.
2. Wipe closure with suitable disinfectant.

Fig. 85-11. Placing an additive into an IV fluid with filtration through a membrane filter (courtesy, Millipore).

3. Insert needle of additive syringe through target area. The vacuum within bottle draws in the solution.
4. Gently shake the bottle after each addition.
5. When completed, cover the closure with a plastic protective cap if it is not to be used immediately.

Travenol/Baxter and McGaw Rigid Glass Containers (Fig. 85-3)

1. Remove the aluminum tear seal and the aluminum disc covering the latex diaphragm.
2. Upon exposing the latex diaphragm, note that the latex cover is drawn in over the openings in the rubber closure.
3. The larger of the two holes receives the administration set, the other is the air vent. The triangle-shaped indentation can serve as the site for injecting the additives as well as the opening for the administration set.
4. Wipe diaphragm with suitable disinfectant and pierce latex cover to place additive into bottle. The vacuum within bottle will draw additive from the syringe. Do not remove diaphragm or the vacuum will dissipate. It will be removed at the time of administration prior to the insertion of the administration set.
5. Gently shake the bottle after each additive.
6. When completed, cover the bottle with a plastic additive cap if administration set is not to be inserted immediately.

Travenol/Baxter and Abbott Plastic Container (Fig. 85-4)

1. Remove additive port protective sleeve and rub gum-rubber plug with suitable disinfectant.
2. Additives are placed in container by piercing gum-rubber cover over the additive port.
3. After each addition, milk the container to insure adequate mixing.
4. Containers do not contain a vacuum, but vacuum chambers are available for use in conjunction with the flexible plastic container.
5. Protective additive caps are available if administration set is not inserted immediately.

McGaw Semirigid Plastic Container (Fig. 85-4)

1. Remove additive port protective covering and rub gum-rubber plug with suitable disinfectant.
2. Additives are placed in containers by piercing gum-rubber over the additive port.
3. After each addition, shake the container gently to insure adequate mixing.
4. Containers do not contain a vacuum.

Parenteral Incompatibility—When one or more additives are combined with an IV fluid, their presence together may modify the inherent characteristics of the drug substances present, resulting in a parenteral incompatibility. Parenteral incompatibilities have been arbitrarily divided into three groups: physical, chemical, and therapeutic. The last are the most difficult to observe because the combination results in undesirable antagonistic or synergistic pharmacological activity. For example, the report that penicillin or cortisone antagonizes the effect of heparin and produces a misleading picture of the anticoagulant effect of heparin represents a therapeutic incompatibility. Physical incompatibilities are the most easily observed and can be detected by changes in the appearance of the admixture, such as a change in color, formation of a precipitate, or evolution of a gas. Physical incompatibilities frequently can be predicted by knowing the chemical characteristics of the drugs involved. For example, the sodium salts of weak acids, such as phenytoin sodium or phenobarbital sodium, precipitate as free acids when added to intravenous fluids having an acidic pH. Calcium salts precipitate when added to an alkaline medium. Injections that require a special diluent for solubilization, such as Valium, precipitate when added to aqueous solutions because of their low water-solubility.

Decomposition of drug substances resulting from combination of parenteral dosage forms is called a chemical incompatibility, an arbitrary classification since physical incompatibilities also result from chemical changes. Most chemical incompatibilities result from hydrolysis, oxidation, reduction, or complexation and can be detected only with a suitable analytical method.

An important factor in causing a parenteral incompatibility is a change in the acid-base environment.[1] The solubility and stability of a drug may vary as the pH of the solution changes. A change in the pH of the solution may be an indication in predicting an incompatibility, especially one involving drug stability, since this is not necessarily apparent physically. The effect of pH on stability is illustrated in the case of penicillin. The antibiotic remains active for 24 hr at pH 6.5, but at pH 3.5 it is destroyed in a short time. Potassium penicillin G contains a citrate buffer and is buffered at pH 6.0 to 6.5 when reconstituted with Sterile Water for Injection, Dextrose Injection, or Sodium Chloride Injection. When this reconstituted solution is added to an intravenous fluid such as Dextrose Injection or Sodium Chloride Injection, the normal acid pH of the solution is buffered at pH 6.0 to 6.5, thus assuring the activity of the antibiotic.

While it may be impossible to predict and prevent all parenteral incompatibilities, their occurrence can be minimized. The IV admixture pharmacist should be cognizant of the increasing body of literature concerning parenteral incompatibilities. This includes compatibility guides published by large-volume parenteral manufacturers;[2,3,4] compatibility studies on individual parenteral products by the manufacturer and published with the product as part of the labeling; the study of the National Coordinating Committee on Large-Volume Parenterals;[5] reference books;[6,7] and literature reports of studies with specific parenteral drugs.[8] The pharmacist should encourage use of as few additives as possible in intravenous fluids since the number of potential problems increases as the number of additives increases. Physicians should be made aware of possible incompatibilities and the pharmacist can suggest alternate approaches to avoid the difficulties. In some instances, incompatibilities can be avoided by selecting another route of administration for one or more of the drugs involved.

Quality Control—Each hospital should have written procedures covering the handling and storage of IV fluids, their use in preparing admixtures, labeling, and transportation to the floors. In-use clarity and sterility tests should be devised to assure that IV admixtures retain the characteristics of sterility and freedom from particulate matter. Training and monitoring personnel involved in preparation of IV admixtures should be done on a regular basis.[9] The efforts of the hospital pharmacy should be no less than those of the industry in following Current Good Manufacturing Practice to assure the safety and efficacy of these compounded medications.

Total Parenteral Nutrition

Intravenous administration of calories, nitrogen, and other nutritients in sufficient quantities to achieve tissue synthesis and anabolism is called total parenteral nutrition (TPN).[10] Originally the term hyperalimentation was used to describe the procedure, but it is being replaced by TPN, the latter being more descriptive for the technique.

The normal calorie requirement for an adult is approximately 2500 per day. If these were to be provided totally by Dextrose Injection 5%, approximately 15 liters would be required. Each liter contains 50 g dextrose, equivalent to 170 calories. However, it is only possible to administer three or four liters per day without causing fluid overload. To reduce this fluid volume the concentration of dextrose would have to be increased. By increasing the dextrose to 25%, it is possible to administer five times the calories in one-fifth the volume. Dextrose Injection 25% is hypertonic and cannot be administered in large amounts into a peripheral vein without sclerosing the vein.

Dudrick developed the technique for administering fluids for total parenteral nutrition by way of the subclavian vein into the superior vena cava where the solution is rapidly diluted by the large volume of blood available, thus minimizing the hypertonicity of the solution. For administration of the TPN fluids, a catheter is inserted and retained in place in the subclavian vein. TPN is indicated in patients who are unable to ingest food due to carcinoma or extensive burns; patients who refuse to eat, as in the case of depressed geriatrics or young patients suffering from anorexia nervosa; and surgical patients who should not be fed orally.

The preferred source for calories in TPN fluids is the carbohydrate dextrose. Both fat emulsions and alcohol are caloric sources, but they are not used in TPN fluids. In IV fluid kits commercially available for the preparation of TPN solutions, Dextrose Injection 50% is provided. On dilution with protein hydrolysate or amino acid injection, the resulting dextrose concentration is approximately 25%. It is this concentration that is administered.

The source of nitrogen in TPN fluids is either protein hydrolysates (*Amigen*®—Travenol; *Aminosol*®—Abbott; *CPH-5*®—Cutter; *Hyprotigen*®—McGaw) or crystalline amino acids (*Aminosyn*®—Abbott; *FreAmine II*®—McGaw; *Travasol*®—Travenol; *Veinamine*®—Cutter). Protein hydrolysates are obtained from casein or fibrin and contain polypeptides which must be broken down before they can be utilized. Although available at lower cost than crystalline amino acids, they contain higher amounts of ammonia and free chloride. In the case of hydrolysates, the exact amount of protein being administered is not known. The crystalline amino acid injections contain all the essential and nonessential amino acids in the L-form. They are more expensive than the protein hydrolysates but contain less ammonia and free chloride. For optimum utilization of amino acids and for promoting tissue regeneration, the nitrogen-to-calorie ratio should be 1:150. Calories are needed to provide energy for the metabolism of nitrogen.

Electrolyte requirements will vary with the individual patient. The electrolytes present in Protein Hydrolysate Injection or Amino Acid Injection are given on the label and must be taken into consideration in determining the quantities to be added. Usual electrolyte concentrations required to fall within the following ranges: sodium, 100–120 mEq; potassium, 80–120 mEq; magnesium, 8–16 mEq; calcium, 5–10 mEq; chloride, 100–120 mEq; and phosphate, 40–60 mEq. It is better to keep a 1:1 ratio between sodium and chloride ions. In adding potassium, the acetate salt is preferred to the chloride. If the combination of calcium and phosphate ions exceeds 20 mEq, precipitation occurs.

In addition to the electrolytes, the daily requirement for both water-soluble and fat-soluble vitamins may be added, usually in the form of a multivitamin infusion concentrate. Iron, folic acid, and vitamin B_{12} should be administered separately from the TPN fluids. Trace elements such as zinc, copper, manganese, and iodide are a concern only in long-term cases and can be added when required.

The Parenteral Prescription

The physician writes an admixture order or parenteral prescription on a physician's order-form located on the patient's chart. A copy of the order is sent to the pharmacy for compounding. It includes the patient's name, room number, the intravenous fluid wanted, additives and their concentrations, rate of flow, starting time, and length of therapy. The order is taken by the technician, nurse, or pharmacist to the pharmacy. Orders may be telephoned to the pharmacy; verification with the original order is made on delivery of the admixture. IV orders are usually written for a 24-hour therapy period; the patient's chart is reviewed daily and new orders are written on a daily basis. The order may be for multiple containers, in which case the containers are numbered consecutively. Unlike the extemporaneously compounded prescription, additives are added without regard to final volume of IV fluid. The prescription is checked for

The clerical work for the admixture is prepared. This includes typing of the label and the preparation of the profile work sheet. The profile sheet is filed so that the pharmacist will be alerted when subsequent containers are due for preparation. Charging the patient's account can be done from the profile work sheet. The label includes the patient's name, room number, bottle number, preparation date, expiration time and date, intravenous fluid and quantity, additives and quantities, total time for infusion, the milliliters per hour, drops per minute, and space for the name of the nurse who hangs the container. The label will be affixed to the container upside down in order that it can be read when hung.

The admixture is prepared by the pharmacist or a supervised technician. In handling sterile products, aseptic techniques as discussed previously must be observed.

Table III—Typical IV Orders (Parenteral Prescriptions)

Prescription	Comment
1. ℞ NSS 1000 ml 125 ml/hr	Sodium Chloride Injection (Normal Saline Solution) 1000 ml, is to be administered at the flow-rate of 125 ml per hour. It will require approximately eight hours.
2. ℞ 1000 D5 NSS + Vits 12 hr	Dextrose Injection 5%, 1000 ml, containing 0.9% sodium chloride and container of vitamin B complex with vitamin C is to be administered over a 12-hour period.
3. ℞ 500 D5 ½NSS KVO	Dextrose Injection 5%, 500 ml, containing 0.45% sodium chloride is to be administered at a rate of flow to keep the vein open (KVO). The flow rate will be approximately 10 ml per hour.
4. ℞ 1000 cc D5/½NSS Add 1 amp Vits to each + 100 mg Thiamine Each to run 6 hr	Dextrose Injection 5%, 1000 ml, containing 0.45% sodium chloride, the contents of one ampul vitamin B complex with vitamin C, and sufficient volume of Thiamine Hydrochloride Injection to give 100 mg thiamine, is to be administered over a 6-hour period (approximately 170 ml per hour). Additional orders of the same can be anticipated.
5. ℞ 1000 cc D5/½ NSS + 20 mEq KCl	Dextrose Injection 5%, 1000 ml, is to be provided containing 0.45% sodium chloride and 20 mEq potassium chloride.
6. ℞ 1000 Hyperal + 10 NaCl + 10 KCl + 5 $MgSO_4$ + 10 Insulin	One liter of the hospital's basic TPN solution is to be provided with the addition of 10 mEq sodium chloride, 10 mEq potassium chloride, 5 mEq magnesium sulfate, and 10 units regular zinc insulin.
7. ℞ 1000 cc Hyperal (FreAmine) + 40 mEq $NaHCO_3$ + 30 mEq KCl + Vits + 5u Reg Insulin to run 80 cc/hr	One liter of the basic TPN solution, FreAmine II, is to be provided with the addition of 40 mEq $NaHCO_3$, 30 mEq potassium chloride, the contents of one container vitamin B complex with vitamin C plus 5 units of regular zinc insulin. It is to be administered at the flow rate of 80 ml per hour (approximately 12 hours).
8. ℞ 1000 Hyperal + 40 mEq NaCl + 10 KCl + 10 Insulin + 10 Cal Gluc.	One liter of the hospital's basic TPN solution is to be provided with the addition of 40 mEq sodium chloride, 10 mEq potassium chloride, 10 units regular zinc insulin, and 10 ml Calcium Gluconate Injection.
9. ℞ Keflin 2 gm 100 ml D_5W q 6 hr	Cephalothin, 2 g, is reconstituted with Sterile Water for Injection and added to a minibottle containing 100 ml Dextrose Injection 5%. This dose is given every 6 hours using a piggyback technique with a flow rate requiring 30 to 60 minutes for delivery.
10. ℞ Gentamicin 80 mg IVPB q 8 hr	Gentamicin, 80 mg, is added to a minibottle containing 100 ml Dextrose Injection 5%. This dose is given every 8 hours using the piggyback technique (IVPB) with a flow-rate requiring at least 80 minutes (not less than 1 mg per minute).

The completed admixture is delivered to the floor. If it is not to be infused immediately (within one hour), it is stored under refrigeration; if refrigerated, it must be used within 24 hours. The nurse checks for accuracy of patient's name, drug and concentration, IV fluid, expiration date, time started, and clarity. The infusion of admixtures can run ahead or behind schedule, necessitating the pharmacist to modify the preparation of continued orders. Examples of IV orders are shown in Table III.

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Human Serum Dipeptidyl Peptidase IV (DPPIV) and Its Unique Properties

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Dipeptidyl peptidase IV (DPPIV, EC 3.4.14.5) has been purified 18,000-fold in a yield of 2.2% from human serum. Serum DPPIV, a serine enzyme with an apparent mass of 250 kDa, consists of two identical subunits with an apparent mass of 100 kDa and is inhibited by DPPIV-specific inhibitor Diprotin A and also by p-chloromercuribenzoate (p-CMB), 2-mercaptoethanol, $HgCl_2$, $CdCl_2$, $SrCl_2$, and $ZnCl_2$. One of the remarkable properties of DPPIV is that its activity is greatly enhanced by Gly-X (X: especially, Gly, Gln, Glu and Ser) dipeptides. Gly-X dipeptides increase not only an apparent Km of serum DPPIV for glycyl-L-proline 3,5-dibromo-4-hydroxyanilide nearly 10-fold, but also an apparent kcat nearly 4-fold. This mechanism is unclear, but one possibility is that Gly-Pro from substrate might bind amino acids or dipeptides instead of water molecules as DPPIV transpeptidyl activity reported previously. Another remarkable property of DPPIV is the ability to bind adenosine deaminase-I and -II, as is the case with recombinant soluble CD26 (rsCD26). This probably indicates that DPPIV purified from human serum by our method originates from T-lymphocytes.

Key words: human serum enzyme, synthetic substrate, glycyl-L-proline derivatives, dipeptides, activator

INTRODUCTION

Dipeptidyl peptidase IV (DPPIV, EC 3.4.14.5) was widely distributed in mammalian tissues (1–3) and fluids (4–6). It was demonstrated by electrophoresis, using fluoro-substrate, 7-glycyl-L-proline 4-methylcoumarinamide, that DPPIV had four isoforms in human serum (7). A main isoform in normal human serum was named peak III, which is consistent with the isoform in human spleen and lymphocytes. DPPIV was also found selectively to occur in T-lymphocytes (8) and reported to be CD26 leukocyte differentiation antigen cluster (9). Subsequently, it was shown that DPPIV/CD26 played an important role in T-lymphocytes activation by the binding of CD3 in the absence of antigen-presenting cells (10) and by the strong binding of adenosine deaminase (11). Recently, one form of human serum DPPIV was purified as a trimer without binding ability to adenosine deaminase (ADA) by Duke-Cohan et al. (12).

We developed a simple colorimetrical rate assay for urinary DPPIV using a new substrate, glycyl-L-proline 3,5-dibromo-4-hydroxyanilide (Gly-Pro-DBAP) (13) and found that urinary DPPIV had the unique property of being activated by some amino acids and dipeptides. Then, we also purified DPPIV from human serum to find out whether it exhibited the same properties as urinary DPPIV. It became clear that purified DPPIV had the same property as urinary DPPIV with respect to its activation by amino acids and dipeptides and that it also had some differences from the DPPIV form purified by Duke-Cohan et al. (12).

MATERIALS AND METHODS

Chemicals

Gly-Pro-DBAP, glycyl-L-hydroxyproline 3,5-dibromo-4-hydroxyanilide (Gly-HyPro-DBAP), and glycyl-azetidine 3,5-dibromo-4-hydroxyanilide (Gly-Aze-DBAP) were synthesized by our method (13). Biririubin oxidase (BO; EC 1.3.3.5) was obtained from Amano Chemical Co. The monoclonal antibody Ta1 (4EL1C7) was purchased from Coulter Immunology, 4H12 from Endogen, BA5 from Serotec, and antimouse CD26, H194-112 from PharMingen. All other chemicals were of analytical grade.

Enzyme Assay

As a standard assay, DPPIV activity was measured using Gly-Pro-DBAP as a substrate in the presence of Glyl-L-Leu

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according to our method (13) in a reaction mixture (total volume 195 µl) containing 0.1 mol/l Bicine buffer pH 9.1, 2 mmol/l p-xylenol, 0.2 U/ml BO, 0.1 mol/l Gly-L-Leu. Kinetic assays were performed using varying concentrations of substrate with and without Gly-L-Leu in order to obtain estimates of an apparent Km and Vmax using Lineweaver-Burk plot. Effects on DPPIV activity were also detected in the presence of anti-DPPIV monoclonal antibodies (50 µg/ml), amino acids, peptides, metal ions, or inhibitors. As a conventional assay, a reaction mixture (total volume 375 µl) containing 2 m mol/l glycyl-L-proline p-nitroanilide (Gly-Pro-pNA) in 0.12 mol/l Tris-HCl buffer pH 8.0 modifying Scherberich's method (14) was used to measure DPPIV activity. The pH optimum was determined using the following buffers; HEPES (pH 6.7–7.6), Bicine (pH 7.5–8.4), CHES (pH 8.2–9.4), and CAPS (pH 8.5–10.3) at final 0.1 mol/l. Enzyme assays were performed in triplet and then the mean values were recorded. ADA activity was measured using a commercial kit, AD Auto MARUHO (Maruho), by the method of Agarwal et al. (15).

SDS-PAGE and Molecular Mass Measurement

The purity was analyzed by the method of Laemmli (16) using a 5–20% gradient SDS-PAGE gel. Staining was performed with Quick-CBB (Wako Pure Chemical Industries), and the enzyme band was determined by molecular mass standards (Daiichi-chemicals) consisting of rabbit muscle myosine (200 kDa), *E. coli* β-galactosidase (116.2 kDa), bovine serum albumin (66.3 kDa), rabbit aldolase (42.4 kDa), bovine carbonic anhydrase (30.0 kDa), and horse myoglobin (17.2 kDa). The molecular mass was determined: (1) on a 1.6 × 60 cm Superdex 200 column (Pharmacia Biotech) measuring DPPIV activity, (2) on a 7.8 × 300 mm TSK gel G3000 SW_{XL} (Tosoh) measuring absorbance at 230 nm, using Bio-Rad molecular mass standards consisting of bovine thyroglobulin (669 kDa), bovine gamma globulin (158 kDa), chicken ovalbumin (44 kDa), equine myoglobulin (17 kDa), and vitamin B-12 (1.35 kDa), and (3) on a 1.0 × 30 cm Superose 6 column (Pharmacia Biotech) measuring DPPIV activity, using Pharmacia Biotech molecular mass standards consisting of bovine thyroglobulin (669 kDa), horse ferritin (440 kDa), bovine catalase (232 kDa), and rabbit aldolase (158 kDa).

Protein Determination

Protein concentration was determined by the bicinchoninic acid-detection reagent (Pierce) using bovine serum albumin as a standard protein.

Binding to Adenosine Deaminases

Modifying the method of Duke-Cohan et al. (12), samples of 25 µg/ml calf intestinal ADA-I and ADA-II were incubated for 30 min at 37°C in the presence or absence of 11 µg/ml purified enzyme in 50 mmol/l Tris-HCl buffer, pH 8.0, 0.25 mol/l NaCl, 0.2% Tween 20, 0.1% NaN_3. The reaction mixtures were measured for DPPIV and ADA activities, and characterized by running on a 7.5% PAGE gel according to the method of Davis (16), followed by silver staining (Wako) with molecular mass standards (Diichi) consisting of bovine thyroglobulin (669 kDa), bovine ferritin (443 kDa), bovine lactate dehydrogenase (139.85 kDa), bovine serum albumin (66.267 kDa), and soy bean inhibitor (20.1 kDa).

Purification of Serum DPPIV

Human serum (400 ml) were prepared from 800 ml of AB blood. After dialysis against 50 mmol/l Tris-HCl buffer, pH 8.0 (buffer A), the protein fraction precipitated by 60–70% saturated ammonium sulfate was applied to a 5 × 30 cm DEAE-FF column, which was washed with buffer A prior to the elution with a linear gradient 0–0.2 mol/l NaCl. The active fractions were pooled and put on a 3.5 × 60 cm Superdex 200 column equilibrated with buffer A. The active fractions were put on a 1.6 × 12 cm Gly-L-Leu bound affinity column, which was prepared with EAH-sepharose 4B (Pharmacia Biotech). After washing the column with buffer A, the enzyme was eluted with a linear gradient 0–0.2 mol/l NaCl. The active fractions were put on a 1 × 2.0 cm Ta1 bound affinity column, which was prepared with EAH-sepharose 4B. After washing the column with buffer A containing 0.5 mol/l NaCl, the enzyme was eluted with 5 mmol/l Tris-HCl buffer, pH 8.0, 2 mol/l NaSCN. The purified enzyme was stored at −80°C, after concentration on a 0.5 × 5 cm DEAE-FF column.

RESULTS

Purification

DPPIV from human serum was purified 18,000-fold in a yield of ~2.2% (Table 1) by sequential purification using DEAE-FF ion exchange chromatography, gel filtration on a Superdex 200 column, a Gly-Leu affinity chromatography, and a Ta1 affinity chromatography. Of total DPPIV activity, 55% was detected in the 60–70% ammonium sulfate fraction. Most of the albumin was separated from DPPIV activity on a DEAE-FF column, but a small amount of albumin was strongly copurified with DPPIV before a Ta1 column. Aminopeptidase M activity, hydrolyzing a substrate Ala-pNA, was also almost copurified with DPPIV up to a Gly-Leu affinity chromatography step, but separated from DPPIV on a Ta1 affinity column. The yield of Ta1 affinity chromatography increased from 10% to 60% after repeated purification. These purification steps were repeated to obtain the requisite amount of DPPIV.

PHYSICOCHEMICAL PROPERTIES

The purified enzyme was detected as a single band on SDS-PAGE (Fig. 1A) and its apparent molecular mass was determined to be 100 kDa, based on a calibration curve prepared

TABLE 1. Purification of Human Serum DPPIV

Step	Total protein mg	Total activity U	Specific activity U/mg	Yield (%)	Purification (-fold)
Serum	23,724	65.6	2.77×10^{-3}	100	1
60–70% Ammonium sulfate fraction	9,141	36.1	3.94×10^{-3}	55.0	1
DEAE-FF	1,102	19.6	1.78×10^{-2}	30.0	6
Superdex 200	255	15.4	6.06×10^{-2}	23.5	22
Gly-Leu affinity	140	12.9	9.28×10^{-2}	19.7	34
Ta1 affinity & DEAE-FF	0.02	1.4	49.7	2.2	18,000

with standard proteins. But the purified enzyme was detected as one main band and three minor bands on PAGE by silver staining (Fig. 1B). A DPPIV active band was detected in consistence with a main band whose molecular mass was determined to be 250 KDa. DPPIV apparent molecular mass was also determined to be 250 and 230 kDa by measuring DPPIV activity on a Superdex 200 and on a Superose 6 column with molecular mass standard proteins, respectively. These results indicated that the serum DPPIV appear to exist as a dimer.

Enzymatic Properties

The purified enzyme showed a pH optimum for the hydrolysis of Gly-Pro-DBAP at about pH 8.5. Antihuman DPPIV/CD26 monoclonal antibodies, Ta1(4EL-1C7), BA5, and 4H12, and antimouse DPPIV/CD26 monoclonal antibody, H194-112, were added to purified enzyme solution, following incubation for 18 hours at 37°C to examine their effects on DPPIV activity. Among them, only the antihuman DPPIV MAb 4H12 inhibited DPPIV activity to 78%. Inhibition of DPPIV activity by DFP and PMSF (Table 2) revealed that serum DPPIV was a serine enzyme.

DPPIV activity was also inhibited by a specific DPPIV inhibitor, Diprotin A. Although inhibited by p-CMB, 2-mercaptoethanol, $HgCl_2$, $CdCl_2$, $SrCl_2$, and $ZnCl_2$, DPPIV activity was activated by several amino acids and peptides in consistency with urinary DPPIV (13) (Table 3). DPPIV activity was activated to ~120% by Gly, Gln, Glu, and Ser, but inhibited to ~50% by Leu, Lys, and Met. In contrast, DPPIV activity was activated by Gly-L-X dipeptides; Gly-Gly, Gly-Leu, Gly-Phe, Gly-Thr, Gly-Gln, Gly-Glu, Gly-Ile, Gly-Ser, and Gly-Tyr, even Gly-Pro, but not or slightly inhibited by Gly-D-Ala, Ala-Ala, and His-Leu (Table 3). It seems that amino acids such as Ala, Arg, Gln, Ile, Leu, Phe, Pro, Ser, and Thr enhanced the activation of DPPIV activity by their amino groups binding the carboxyl group of glycine. It was

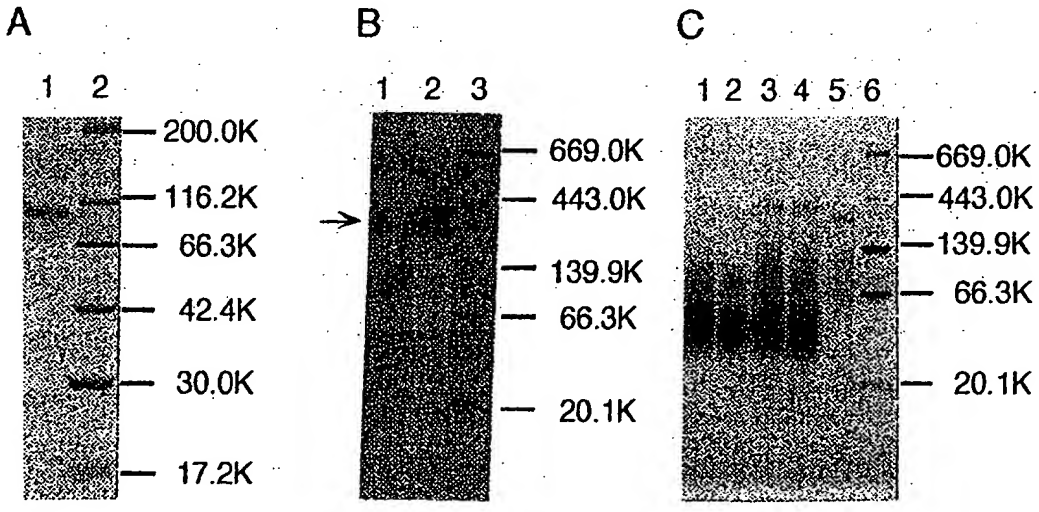

Fig. 1. PAGE and SDS-PAGE of DPPIV and ADAs. A. Analysis of DPPIV (7 μg) purified from human serum (lane 1) and molecular mass markers (lane 2) by SDS-PAGE (5–20% gradient gel) under reducing conditions. B. Analysis on Tris-glycine native PAGE (7.5%) of DPPIV (0.2 μg) purified from human serum by protein (lane 1) and activity staining (lane 2), and by molecular mass markers (lane 3). C. Analysis on PAGE of DPPIV (0.1 μg) without (lane 5) and with ADA-I (lane 4) and ADA-II (lane 3), and analysis of 0.25 μg of ADA-I (lane 2) and 0.25 μg of ADA-II (lane 1), and of molecular mass markers (lane 6).

TABLE 2. Effect of Metal Ions and Inhibitors on DPPIV Activity

Compound	Conc. (mmol/l)	Relative activity (%)
None	–	100
HgCl2	1	59
CdCl2	1	58
SrCl2	1	85
CuCl2	1	93
BaCl2	1	99
NiCl2	1	99
CaCl2	1	99
MgCl2	1	99
CoCl2	1	101
ZnCl2	1	74
EDTA	1	100
2-Mercaptoethanol	1	69
DTT	1	98
Iodoacetaate	1	97
p-CMB	1	61
Benzamidine	1	101
PMSF	1	84
DFP	14	1
DiprotinA	1	83
Antipain	1	102
Elastatinal	1	99
Pepstatin	1	102
Phosphoramidon	1	101
AlphamenineA	1	102
AlphamenineB	1	102
TLCK	1	94
E-64	1	102
Trypsine inhibitor (egg white)	1	104
Bestatin	1	103
Chymostatin	1 mg/ml	94
Leupeptin	1 mg/ml	100

TABLE 3. Effect of Amino Acids and Dipeptides on DPPIV Activity

Compound amino acid	Relative activity (%)	Compound dipeptide	Relative activity (%)
None	109	None	100
Ala	109	Gly-Ala	134
		Gly-D-Ala	105
Arg	107	Gly-Arg	124
Asp	94	Gly-Asp	96
		Gly-Asn	122
Gly	123	Gly-Gly	127
Gln	121	Gly-Gln	157
Glu	119	Gly-Glu	112
Ile	100	Gly-Ile	155
His	93	Gly-His	95
Leu	57	Gly-Leu	139
Lys	59		
Met	52		
Phe	105	Gly-Phe	138
Pro	90	Gly-Pro	130
Ser	118	Gly-Ser	137
Thr	109	Gly-Thr	152
Val	108		
HyPro	91		
		Gly-Tyr	139
		Ala-Ala	85
		His-Leu	55
		Phe-Phe*	92
		Gly-OMe.HCl	127
		NAc-Gly	91

Final concentration of amino acids and dipeptides is 0.1 mol/l, but that of Phe-Phe* is 0.01 mol/l.

also activated by glycine methylester, but not by N-acetyl glycine. DPPIV activity of both serum and urine was maximally activated to 157% at 0.1 mol/l Gly-Ile (Fig. 2). Gly, Gly-Gly and Gly-Gly-Gly also activated DPPIV activity to a peak at 0.1 mol/l (data not shown).

Table 4 shows the substrate specificity of DPPIV purified from human serum. Gly-Aze-DBAP and Gly-HyPro-DBAP were synthesized as substrates containing azetizine and hydroxyproline as proline analogs. Replacement of Pro by azetizine resulted in higher hydrolysis by DPPIV, whereas replacement of Pro by hydroxyproline resulted in lower hydrolysis by DPPIV. Hydrolysis of their substrates by DPPIV was inhibited by Gly-Leu, which activated hydrolysis of Gly-Pro-DBAP. Substrates containing p-nitroaniline as a chromophore group were used at 2 mmol/l in the reaction mixture at pH 8 because they were very sensitive to nonenzymatic hydrolysis. No hydrolysis of Leu-pNA, Ala-pNA, Succinyl-Gly-Pro-pNA, and Gly-Arg-pNA was observed. It was revealed that purified DPPIV from human serum was not contaminated with aminopeptidase M (EC 3.4.11.2), amino peptidase P (EC 3.4.11.9) and prolyl oligopeptidase (EC

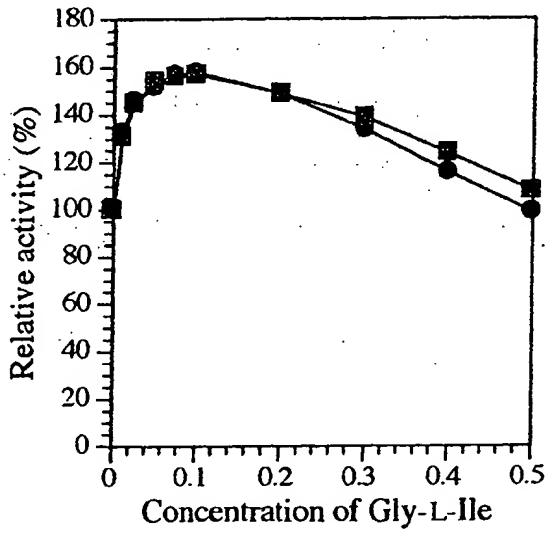

Fig. 2. Effect of concentration of Gly-L-Ile on DPPIV activity. Close circles show DPPIV activity from serum and close squares in urine.

TABLE 4. Specificity of Serum DPPIV With and Without Dipeptides

Substrate	Dipeptides	Relative activity (%)
Gly-Pro-DBAP	None	100
	Gly-L-Leu	139
Gly-Aze-DBAP	None	681
	Gly-L-Leu	512
Gly-HyPro-DBAP	None	24
	Gly-L-Leu	7
Gly-Pro-pNA	None	46
Leu-pNA	None	0
Ala-pNA	None	0
Succinyl-Gly-Pro-pNA	None	0
Gly-Arg-pNA	None	0
Val-Ala-pNA	None	6

3.4.21.26). Table 5 summarizes the remarkable effects of dipeptides on the kinetic parameters of DPPIV analyzed by Lineweaver-Burk plots. An apparent Km of DPPIV for Gly-Pro-DBAP without dipeptides was 0.17 mmol/l. Its value increased to 1.46 mmol/l with Gly-Gly, 1.02 mmol/l with Gly-Leu, and 0.95 mmol/l with Gly-Gln. Surprisingly, kcat also increased from 810 without dipeptides to 3270 with Gly-Gly, 2010 with Gly-Leu and 2770 with Gly-Gln.

Association of Serum DPPIV With ADA-I and ADA-II

The remarkable result obtained was that only the main band with DPPIV activity at 250 kDa disappeared, and a new broad band appeared at 280 ~ 350 kDa by incubations with ADA-I or ADA-II (Fig. 1C), without altering either DPPIV nor ADA activities during incubation with DPPIV and ADAs. This result suggests that 1–3 molecules of ADA could associate with one molecule of DPPIV without interfering with each activity. The fact that there is no difference in molecular mass of their complex with ADA-I and ADA-II indicates that both ADA-I and ADA-II can associate with DPPIV in the same manner.

DISCUSSION

Soluble DPPIV has been purified from human serum to high purity by Ta1 affinity chromatography. Ta1 was selected among DPPIV/CD26 monoclonal antibodies because it had no inhibition against DPPIV activity and its affinity chromatography could produce purer enzyme preparation with higher yield. Although contaminated with few amounts of protein detected by silver staining, a main band with a mass of 250 kDa comigrated with DPPIV active band on PAGE, suggesting that serum DPPIV could exist as a dimer as previously reported for DPPIV from other human sources (1–5,18). The molecular mass of DPPIV monomer was determined on SDS-PAGE to be 100 kDa, suggesting a close correspondence not only with DPPIV from other sources (2–5), but also with recombinant soluble CD26 (12). Inhibition by several metal ions, Hg^{2+}, Cd^{2+} and Zn^{2+}, and p-CMB, exhibited similar properties as DPPIV from other origins (1–5), especially lymphocytes (18). This evidence suggests that DPPIV purified by our method might be originated from T-lymphocytes and have an important function in relation to its activation. Further evidence is provided by PAGE data that purified DPPIV was associated with ADA-I and ADA-II. Analysis for N-sequence of purified DPPIV remains to be compared with rsCD26.

It is a very interesting and unique property that DPPIV activity is activated by several amino acids and peptides, since dipeptides such as Gly-Pro and Gly-Leu are reported to be competitive inhibitors (19). It was previously reported that urinary DPPIV activity was activated by several amino acids and peptides (13). Moreover, this report notes that DPPIV purified from serum is not only activated by several aminoacids and peptides, but also by glycine methylester. Comparison data between amino acids and Gly-X dipeptides suggest that the esterification of glycine carboxyl group with methyl group and the acylation with amino-terminals of several amino acids enhance the activation of DPPIV activity. It is possible that carboxyl terminals in amino acids and peptides may electrostatically interact with catalytic and/or binding sites. It is unclear why DPPIV activity is activated by high concentrations of amino acids and dipeptides. One possibility is that Gly-Pro released from the substrate by DPPIV might bind to amino acids or dipeptides instead of water molecule, as supported by a report that DPPIV has transpeptidyl activity (20). Moreover, it is possible that the transpeptidyl activity of DPPIV might have physiologic function in areas such as intestinal and renal brush border membranes, where amino acids and peptides are found in high concentrations.

TABLE 5. Kinetic Parameters of Serum DPPIV

Substrate	Dipeptides	Km ($mol \cdot 10^{-3} \cdot l^{-1}$)	kcat (s^{-1})	kcat/Km ($s^{-1} \cdot l \cdot 10^{3} \cdot mol^{-1}$)
Gly-Pro-DBAP	None	0.17	810	4,765
	Gly-Gly	1.46	3,270	2,240
	Gly-L-Leu	1.02	2,010	1,971
	Gly-L-Gln	0.95	2,770	2,916

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